Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8999–9003, August 1996
Cell Biology

Alteration of myosin cross bridges by phosphorylation of myosin-binding protein C in cardiac muscle

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Communicated by Robert E. Forster, University of Pennsylvania Medical Center, Philadelphia, PA, May 10, 1996 (received for review December 4, 1995)

ABSTRACT **In addition to the contractile proteins actin and myosin, contractile filaments of striated muscle contain other proteins that are important for regulating the structure and the interaction of the two force-generating proteins. In the thin filaments, troponin and tropomyosin form a Ca-sensitive trigger that activates normal contraction when intracellular Ca is elevated. In the thick filament, there are several myosin-binding proteins whose functions are unclear. Among these is the myosin-binding protein C (MBP-C). The cardiac isoform contains four phosphorylation sites under the control of cAMP and calmodulin-regulated kinases, whereas the skeletal isoform contains only one such site, suggesting that phosphorylation in cardiac muscle has a specific regulatory function. We isolated natural thick filaments from cardiac muscle and, using electron microscopy and optical diffraction, determined the effect of phosphorylation of MBP-C on cross bridges. The thickness of the filaments that had been treated with protein kinase A was increased where cross bridges were present. No change occurred in the central bare zone that is devoid of cross bridges. The intensity of the reflections along the 43-nm layer line, which is primarily due to the helical array of cross bridges, was increased, and the distance of the first peak reflection from the meridian along the 43-nm layer line was decreased. The results indicate that phosphorylation of MBP-C (*i*) extends the cross bridges from the backbone of the filament and (*ii*) increases their degree of order and/or alters their orientation. These changes could alter rate constants for attachment to and detachment from the thin filament and thereby modify force production in activated cardiac muscle.**

Although force and movement in muscle cells are generated by an interaction between actin and myosin, there are other proteins in the thick and thin contractile filaments that may play an important role in contraction. In the thin filament, troponin and tropomyosin act as a regulatory unit that maintains relaxation in striated muscle. In the normal contraction, this inhibition of interaction between actin and myosin is relieved as Ca is bound to a subunit of troponin.

There are several proteins in the thick filament in addition to myosin. Some data suggest that they may be important in the formation and maintenance of the structure of the filament and possibly the myofibril, but they also may contribute to the modulation of the contractile performance of the mature cell. Myosin-binding protein C (MBP-C, also called C protein in the literature) is of particular interest in the heart because it can be phosphorylated by two different kinases, leading to a total of four phosphorylation sites per protein (1–4). One kinase, which is bound to the thick filament itself and generally stays with the thick filament during isolation of myofibrils and filaments, is regulated by the combination of Ca and calmodulin. At least one of the four phosphorylation sites can only be phosphorylated by this calmodulin-sensitive enzyme (Ca/calmodulin kinase, CAMK). The other three sites can be phosphorylated by a cAMP-regulated kinase (PKA). Recent studies of cardiac MBP-C using mutagenesis suggest that a hierarchical arrangement exists among the phosphorylation sites. The CAMK-regulated site must be phosphorylated first before the PKA-regulated sites can be phosphorylated. All of this is of particular interest in cardiac muscle, because the skeletal isoform of MBP-C contains only one phosphorylation site, and the contractile apparatus in cardiac muscle seems to be much more plastic in its functional properties than skeletal muscle.

Isometric force, the rate of rise in tension, the rate of fall of tension, and the Ca sensitivity of the activation process can be regulated in cardiac muscle by changes in Ca cycling and/or phosphorylation of the contractile proteins. The molecular bases for these forms of regulation are not well understood, and for the β-adrenergic agonists, the data are conflicting. The velocity of unloaded shortening, the cycling of the cross bridges, and the ATPase activity of actomyosin have been reported as increased, unchanged, and under certain conditions even decreased (5–9). The effect of cAMP and PKA on actomyosin ATPase activity appears to be modulated by a second factor that may, under physiological conditions, be released by endothelial cells and cause its effect through protein kinase C (PKC). Venema and Kuo (10) have shown that PKA- and PKC-mediated phosphorylation of cardiac myofibrils have opposite effects on actomyosin ATPase activity through different phosphorylations of troponin in conjunction with similar phosphorylation of MBP-C.

Phosphorylation of MBP-C, although apparently the same with both PKA and PKC, may be important in producing the effects of each of the two kinases on the myofibrils. Under certain conditions where actomyosin ATPase activity has been increased and decreased by the combination of α- and β-adrenergic agonists, there is a good correlation between enzymatic activity and the degree of phosphorylation of MBP-C. No correlation was found with phosphorylation of the regulatory light chain of myosin (LC2) or troponin.

When actomyosin ATPase activity has been increased by PKA, there is a smaller but consistent increase in the Ca-activated ATPase activity of myosin, which does not involve any interaction with actin (11). MBP-C is the only thick filament protein phosphorylated by the kinase (9), indicating that the phosphorylation of MBP-C produces some change in myosin at its ATPase site in the cross bridge region of the molecule.

To see if phosphorylation of MBP-C produces any structural correlate to the change in myosin ATPase activity, native thick filaments were isolated from rat ventricle, and the effect of PKA-mediated phosphorylation of MBP-C on the structure of the thick filament was determined by negative staining and optical diffraction. The results show that the phosphorylation results in an extension of the cross bridge from the backbone of the thick filament by an amount that would place it at the surface of the thin filament in the intact filament lattice. This

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Abbreviations: MBP-C, myosin-binding protein C; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.

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change would appear to put the cross bridge in a more favorable position for attachment to actin.

METHODS

Isolated thick filaments were prepared by a modification of the method of Kensler and Levine (12). Hearts were dissected in oxygenated Krebs' solution from euthyroid rats weighing 175–600 g. The ventricles were opened, pinned out on Sylgard, and allowed to remain in oxygenated incubation solution (50 mM NaCl/2.5 mM magnesium acetate/1.0 mM EGTA/5 mM imidazole/0.5 mM DTT/5.0 mM ATP/0.2 mM sodium azide plus a protease mixture containing 0.001 nM phenylmethylsulfonyl fluoride, L-1-tosylamido-2-phenylethyl chloromethyl ketone at 0.1 mg/liter, N^{α}-benzoyl-L-arginine methyl ester at 0.1 mg/liter, benzamidine at 0.05 mg/liter, aprotinin at 0.01 ml/liter, leupeptin at 0.02 mg/liter, and pepstatin at 0.05 ng/500 μl) for 1 hr at room temperature. The ventricles were then frayed with a sharp scalpel. The tissue with its solution was placed into a refrigerator at 4°C overnight. The following morning the tissue was transferred to 200 μl of fresh incubation solution to which 80 μl of soybean trypsin inhibitor (2 mg/ml) and 80 μl of elastase (2 mg/ml) had been added at room temperature. After 3 min the tissue was vigorously agitated by shaking and repeated pipeting. The solution with the tissue was centrifuged in a Fisher microcentrifuge (model 59A) for 3 min at a setting of 2.5 (out of 10). The pellet was resuspended in 150 μl of the same solution and divided into two parts for control and protein kinase treatment. Calmidazolin, calyculin A, and cAMP were added to each of the two solutions to a final concentration of 10 μM, 100 μM, and 10 μM, respectively. The calmidazolin and calyculin inhibit, respectively, calmodulin and phosphatase. PKA from bovine heart (Sigma) was added to one of the two solutions to a final concentration of 100 μg/ml of solution. One solution contained ATP, PKA, and cAMP to phosphorylate MBP-C, EGTA and calmidazolin to prevent phosphorylation of the regulatory light chain of myosin (LC2), and calyculin to block phosphatase, while the other solution did not contain PKA or cAMP. The control and phosphorylating solutions were incubated in a shaking water bath at 30°C for 30 min. They were then centrifuged in the microcentrifuge for 3 min and resuspended in solution without the PKA or calmidazolin, and 20 μl of suspension was placed on carbon-coated grids and washed five times with the same solutions in which they had been suspended. The fluid was removed each time by absorption into a filter paper. The grids were negatively stained in 1% uranyl acetate plus one drop of glycerol for 30 sec. The uranyl acetate solution was removed by wicking off most of the stain and allowing the remaining liquid to evaporate in air at room temperature.

Thick filaments lying on the carbon over the holes in the grids were viewed with a JEOL transmission electron microscope and photographed at ×20,000. The electron micrograph negatives were diffracted in a laser optical diffractometer (2-mW helium/neon, 632.8 nm) using a diffractometer camera that has been calibrated from diffraction patterns of micrographs of catalase crystals. Measurements of diffraction pattern spacings were made on large prints of the transform.

The relative intensity of the first-order reflection was determined by comparing its intensity with the average intensity of the reflections along the meridian between 43 and 14 nm. The procedure was chosen ito provide an internal standard in each film to avoid variations from different batches of film or developing reagents. A direct comparison of the intensity of the reflection among the optical diffraction patterns gave similar results as long as the exposure and development times were the same.

For the measurement of the thickness of filaments, only those with clear central bare zones, a length of 1.6 μm, tapered ends, and visible periodicity were chosen. The electron micrographs were visualized through a microscope with a calibrated scale in one eyepiece, and the thickness of the filament at several points was measured. The measurements were made independently by two people. Reproducibility was ±1.0 nm.

RESULTS

The majority of isolated filaments had well preserved structure (Fig. 1). They were 1.6 μm long with tapered ends and a smooth thinner region of about 0.15 μm in the center of the filaments corresponding to the segment that has no cross bridges. Outside of this central zone, the filaments were thicker and contained regions with discernible periodicity. These observations indicated that the full length of the thick filaments was intact and the central bare zone was retained. Specific phosphorylation of MBP-C by cAMP-activated PKA and the absence of phosphorylation of LC2 were demonstrated by using radioactive ATP with PKA and cAMP and examining autoradiographs of gels after electrophoresis of the proteins in the thick filament preparation (Fig. 2). In kinase-treated but not in control filaments, substantial ^{32}P was present in a band at 150 kDa, the position of MBP-C. There was no significant radioactivity at 24 or 19 kDa, the locations of the light chains of myosin.

Measurements were made of the thickness of the central bare zones and the regions of more than 150 control and 150 kinase-treated filaments where the periodic structures could clearly be defined. These filaments were from nine hearts and satisfied several requirements. The filaments were complete with central bare zones, a length of approximately 1.6 μm, tapered ends, and visible periodicity. The average periodicity among these regions was 14.4 ± 1.5 nm (SD), which agrees very well with the periodicity of cross bridges that has been determined by x-ray diffraction and electron microscopy (13, 14). The thickness of the bare zones was uniform with the same mean values and standard deviations in the control and the kinase-treated filaments [20.1 ± 0.8 nm (SD)]. On the other hand the thickness where cross bridges were identifiable was greater in

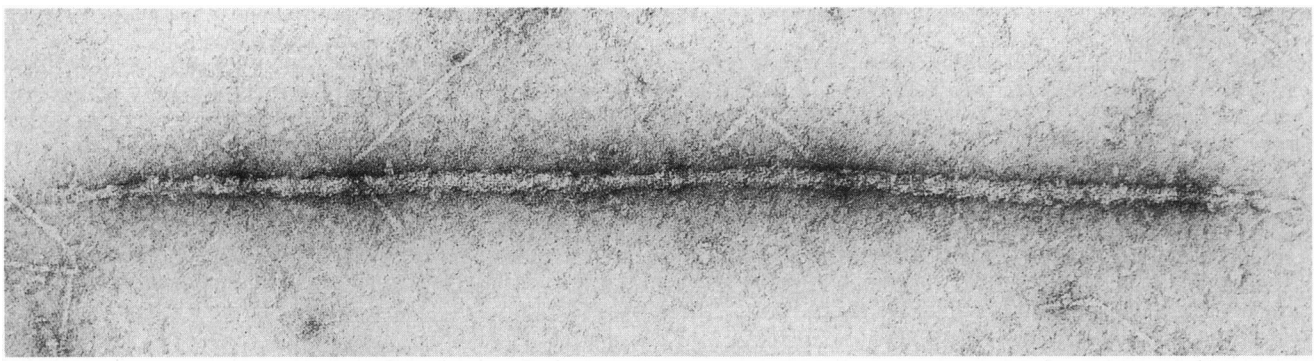

FIG. 1. Electron micrograph of a negatively stained thick filament showing periodicity of cross bridges, central bare zone, and tapered ends.

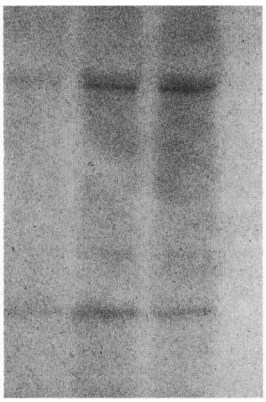

FIG. 2. Autoradiograph of a gel after polyacrylamide gel electrophoresis of preparations of isolated natural thick filaments from rat ventricle. The lanes from left to right show, respectively, control with no added PKA and cAMP and two separate preparations with PKA and cAMP present. The autoradiograph shows ^{32}P incorporation into a band at approximately 150 kDa, where MBP-C would be expected, and at 29 kDa, where TNI would be expected (from thin filaments, which do not interfere with optical diffraction or measurements of thick filaments). There is no ^{32}P incorporation at either 24 or 19 kDa, where the regulatory and essential light chains of myosin should be.

the kinase-treated than in the control filaments (32.8 ± 1.4 nm in control versus 35.0 ± 1.1 nm in the kinase-treated); $P < 0.02$). The distribution of the thicknesses of the two sets of filaments is shown in histograms in Fig. 3. The control filaments consisted of two populations. The two populations were not due to the presence of α and β myosin heavy chain (MHC) in different cross bridges because the distributions were the same in hearts from 175-g rats, which contain essentially only α MHC, and 600-g rats, which contain about 15% β MHC. Most had a mean thickness of near 30 nm, but a minority, about 28%, fell about a second mean of approximately 37 nm. All of the kinase-treated filaments distributed around a single mean of approximately 36 nm, which was not significantly different from the 37 nm in the control filaments. These results indicate that phosphorylation of MBP-C produces a population of cross bridges that extend a near uniform distance from the backbone of the thick filament. In the control filaments, most of the filaments had cross bridges about 3 nm closer to the backbone. The remainder extend the same distance as the cross bridges in the kinase-treated filaments. The latter are probably associated with phosphorylated MBP-C in the control filament. MBP-C isolated from cardiac muscle by the usual procedures contains about one phosphate per molecule (refs. 3, 4, and 10 and results not shown) or about 25% of maximum phosphorylation, not significantly different from the fraction of cross bridges in control filaments that lie at a greater distance from the backbone of the thick filament.

Electron micrographs were illuminated with laser light, and the diffraction pattern was recorded. In a majority of filaments, reflections along the 43-nm layer line were clearly visible (Fig. 4). Sometimes as many as three peaks were present along the layer line. Because the deposition of stain in the negative staining process was not always uniform, symmetrical reflections were sometimes not seen in all four quadrants of the diffraction pattern, but diagonal symmetry was always present. Sometimes there was a meridional reflection at 14 nm, and occasionally a satellite reflection along the 14-nm layer line was also seen.

Two consistent differences were observed in the diffraction patterns of the control and the kinase-treated filaments among the more than 500 filaments from nine hearts that were studied. In kinase-treated filaments, along the 43-nm layer line, the first maximum (corresponding to the third-order Bessel function {8}) was always closer to the meridian. The 43-nm reflection is produced primarily by the helical arrangement of cross bridges

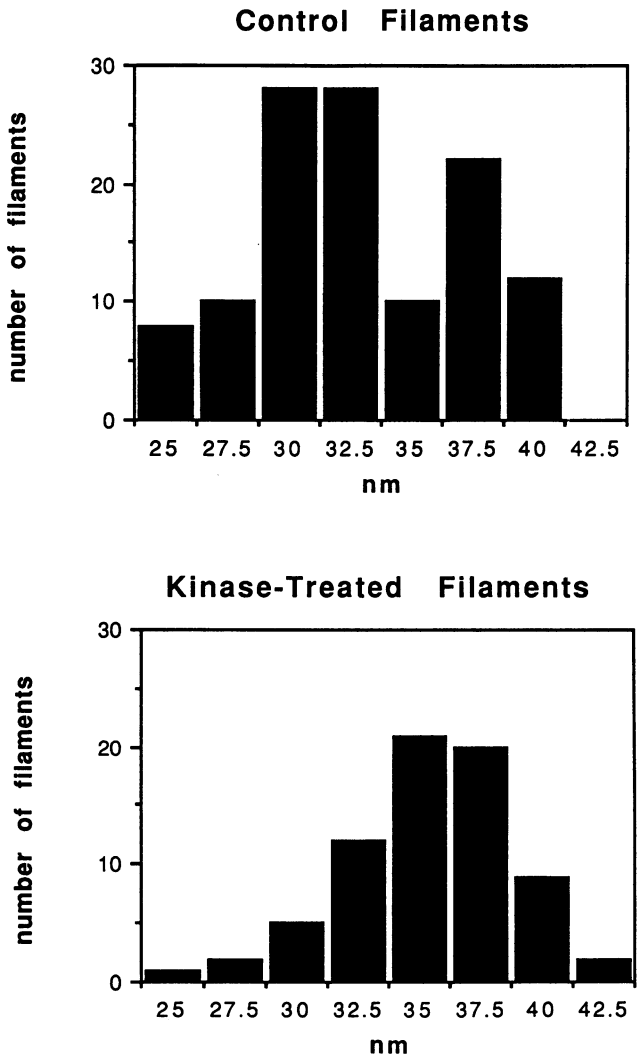

FIG. 3. Histograms of the thickness of isolated thick filaments at regions where cross bridge periodicity was clearly visible. Each filament was placed in the appropriate one of a series of bins that differed by 2.5 nm. (*Upper*) Control. (*Lower*) Kinase-treated. The distributions are significantly different at $P < 0.001$.

along the thick filament and by the presence of MBP-C at 43-nm intervals along regions of thick filament in the central third of each half of the filament (14). In patterns from isolated thick filaments, this is not a lattice reflection. The location of the first-order reflection with respect to the meridian can be used to calculate the location of the center of mass of the cross bridges with respect to the axis of the thick filament, assuming concentration of its mass at a single point (12). In the filaments that had not been exposed to PKA and cAMP, the center of mass of the cross bridges was 11.5 ± 1.3 nm (SD) from the axis of the thick filament. In filaments with phosphorylation of MBP-C, the center of mass of cross bridges was significantly farther from the axis of the thick filament, at 13.8 ± 0.9 nm (SD) ($P < 0.01$).

The intensity of the reflections along the 43-nm layer line was greater in the phosphorylated filaments. The intensity of the first-order reflection along the 43-nm layer line was normalized to the average intensity of other reflections within the diffraction patterns and expressed as a ratio. These ratios from the individual control and kinase-treated filaments from each heart were averaged and then compared with each other (Fig. 5). In eight of the nine hearts that were studied, the intensity of the reflection was greater in the phosphorylated than the nonphosphorylated filaments. In the one exception, the intensity of the control was high, and the thickness of the

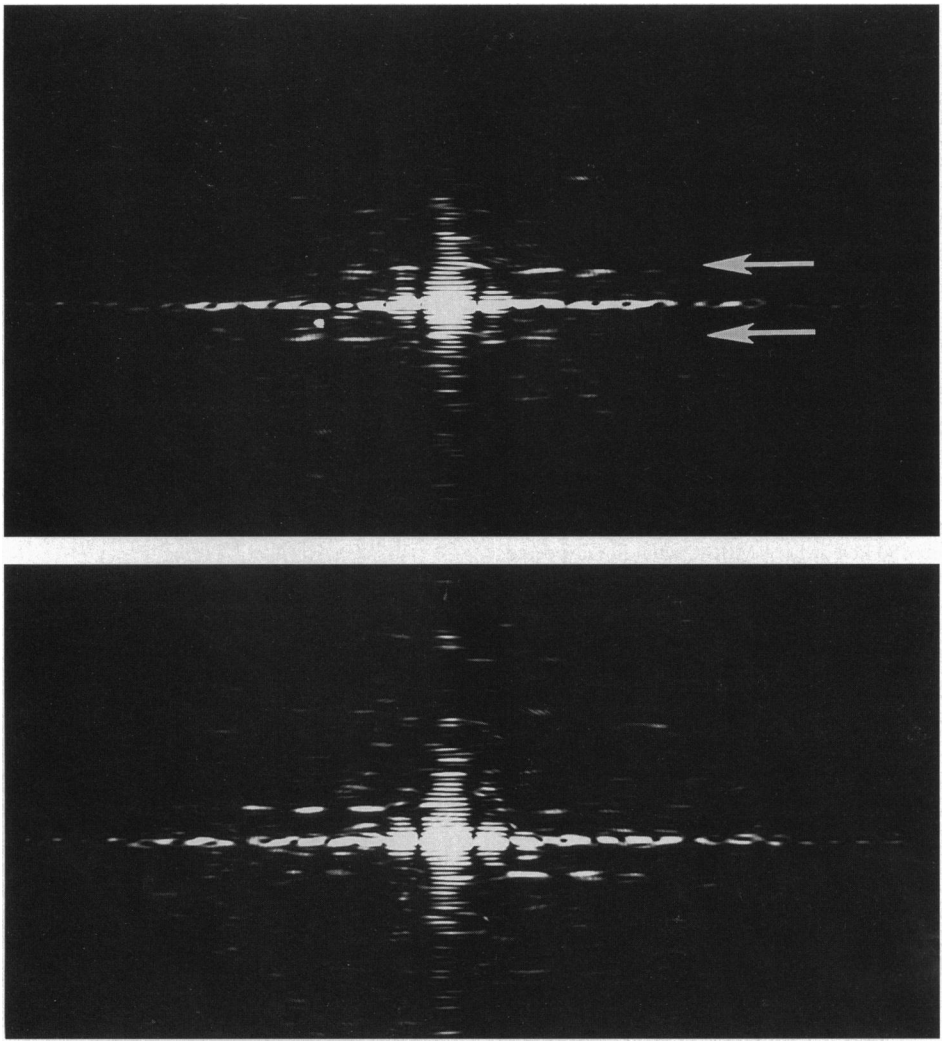

FIG. 4. (*Upper*) Optical diffraction from a negatively stained thick filament that has not been exposed to PKA and cAMP (control filament). Reflections along the 43-nm layer line are visible (marker). (*Lower*) Optical diffraction pattern from a negatively stained thick filament that had been exposed to PKA and cAMP to produce phosphorylation of MBP-C. The reflections along the 43-nm layer line are stronger than in the control filament shown in *Upper*. The first-order reflection lies closer to the meridian than in the diffraction pattern in the control filament shown in *Upper*.

cross bridges in the control had the pattern of kinase-treated filaments. There was some variation in the values for the nonphosphorylated filaments, but little difference existed among all but two of the phosphorylated hearts. These results can be explained by an increase in order or a change in the orientation of the cross bridges in thick filaments with phosphorylated MBP-C. Variability in the degree of order of the cross bridges, presumably from different levels of MBP-C phosphorylation, is eliminated by exposure to activated PKA and a resultant increase in phosphorylation. The same conclusion can be drawn from the distribution of filament thickness in the control and the kinase-treated filaments. In the ninth heart, mentioned as an exception above, the two values for the intensity of the reflection were not significantly different presumably because the control had cross bridges extended as with phosphorylated filaments. This could be explained by a high level of phosphorylation in the control.

As a control, isolated thick filaments from two additional hearts were exposed to an inactivated form of PKA with cAMP. No difference was observed in the diffraction patterns produced by control filaments and filaments exposed to the inactivated PKA, and the intensity of the first-order reflection along the 43-nm layer line was similar to those found in control filaments from the same preparations.

DISCUSSION

The values for the thickness of the thick filaments and the distance of the center of mass of the cross bridges from the filament axis are both very similar to those found by Kensler and Stewart (15) in skeletal muscle. They are about 20% smaller than Kensler and Levine (12) found in the Limulus thick filaments, but the extension of the cross bridge is about the same in control cardiac and Limulus filaments. This difference is consistent with filaments in mammalian cardiac muscle being three-stranded compared with the four-stranded Limulus filaments. In control filaments the center of mass of the cross bridge, presumably about 4.5 nm from the actin binding site at the end of the cross bridge (16), lies only 1.5 nm from the backbone of the thick filament as calculated from the diffraction data. In the kinase-treated filaments with enhanced phosphorylation of MBP-C, the center of mass is extended from the backbone of the thick filament an additional 2.3 nm to a value of 3.8 nm. This is very similar to the change in the location of the end of the cross bridge that has been estimated from the changes in the histograms of the thickness of the filament. The movements of the end and the center of mass indicate that the entire head of the myosin molecule is probably shifted by a change in the conformation of the stalk of the S1 portion of the molecule, which contains the two light chains (16). This is believed to be the more

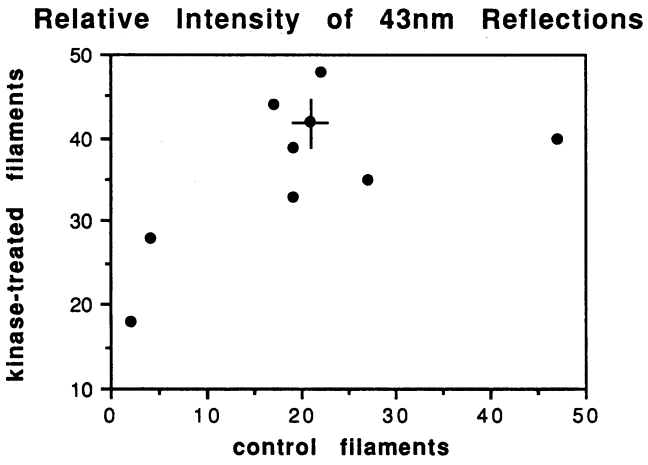

FIG. 5. Relation between the relative intensity of the first-order reflection on the 43-nm layer line in control filaments (abscissa) and filaments that had been exposed to PKA and cAMP to produce phosphorylation of MBP-C (ordinate). Each point represents the results of 50–80 filaments from a single heart. The SEMs are shown for one representative point. Errors in the other points are very similar but are not shown to avoid congestion of the graph.

compliant portion of the cross bridge that rotates during length changes of striated muscle (17).

The center to center distance between adjacent thick and thin filaments in intact myocytes from mammalian heart is approximately 23 nm (18). Using the dimensions of myosin and the positions of the center of mass and the site that attaches to actin from the x-ray crystallographic model of Rayment *et al.* (16), one can estimate the relative change in distances between myosin and actin interacting sites that phosphorylation of MBP-C would produce in the filament lattice in the intact cell at physiological sarcomere length (Fig. 6). In the control state, the average position of the actin-binding site would lie about 3 nm from the thin filament. Phosphorylation of the MBP-C would extend the cross bridge to the surface of the thin filament. It is not possible from the data to determine whether the orientation of the actin-binding site with respect to the attachment site on actin is altered as well, but this does seem to be likely. It is quite reasonable that the rates of attachment and detachment of the cross bridges to actin are modified by the change in the positon of the cross bridge.

During the period of relaxation between rhythmic contractions cross bridges lie close to the thin filaments, but the cross bridges are much closer to the thick filaments during prolonged relaxation in a quiescent heart (18). This phenomenon can be reproduced in

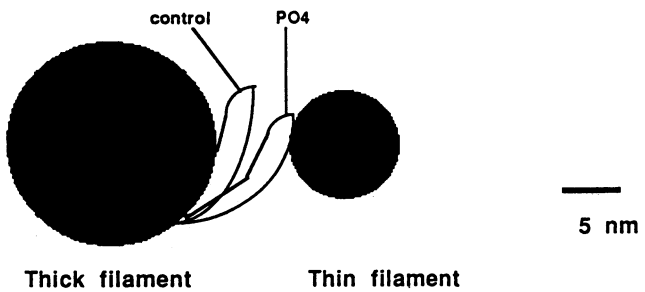

FIG. 6. Diagram drawn approximately to scale showing the effect of the change in the position of the cross bridge from phosphorylation of MBP-C on the distance of the actin-binding site from the thin filament.

skinned cardiac fibers by changing the Ca concentration (19). At Ca concentration much below the threshold for activation of contraction, cross bridges lie close to the backbone of the thick filament, but raising Ca concentration to slightly above the level required to activate contraction shifts about 80% of the cross bridge mass toward the thin filament. This does not happen in skeletal muscle in which the isoform of MBP-C is different and contains only one phosphorylation site.

Genetic studies of cardiac MBP-C (2) have shown that its phosphorylation is complex. One of the four sites is specific for a Ca/calmodulin-regulated kinase that is bound to MBP-C, and phosphorylation of this site is required for the phosphorylation of other sites, which are PKA controlled (2, 3). Thus with the results reported here and the x-ray diffraction data of Matsubara and Millman (18, 19), it appears that phosphorylation of MBP-C may play an important role in regulating the position of the myosin head with respect to the thin filament. This in turn is quite likely to have an influence on the kinetics of the interaction between myosin and actin and the generation of force.

Recent results (20) indicate that endothelin can modify the efficiency of contraction in cardiac muscle by altering the kinetics of the cross bridge cyclical interaction with actin. In view of the indirect evidence that endothelin raises intracellular Ca (21) and activates PKC (22), which itself can phosphorylate MBP-C (10), it is tempting to relate the observations on endothelin to the effect of phosphorylation on cross bridges. The mechanism by which endothelin can alter cross bridge kinetics may be phosphorylation of MBP-C. Such a mechanism would be unique to cardiac among the mammalian striated muscles because only cardiac MBP-C contains the necessary sequences for phosphorylation (2, 23).

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